Original article

Peripheral blood T cell proliferative response to chlamydial organisms in gonococcal and non-gonococcal urethritis and presumed pelvic inflammatory disease

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Objective: To study peripheral blood mononuclear cell (PBMC) proliferative response to *Chlamydia trachomatis* elementary bodies in (a) controls, (b) various stages of gonococcal (c) and non-gonococcal urethritis, and (d) women with a clinical diagnosis of pelvic inflammatory disease (PID).
Methods: We categorised 102 men presenting to a GUM clinic with urethritis by organisms (*C trachomatis* (CT) or *Neisseria gonorrhoeae* (NG) (both by culture), and whether it was their first (urethritis naive) or subsequent (urethritis experienced) attack. 23 women presenting to the clinic with a clinical diagnosis of PID were also investigated. We measured PBMC proliferative responses to *C trachomatis* (DK20—an oculogenital strain, serovar E), lysate of McCoy cells (used to propagate chlamydiae), and the recall antigen PPD. Controls were 37 men and women without present or past history of urethritis or chlamydial infection. Results were expressed as the ratio of the stimulation index (SI) obtained with DK20 compared with McCoy cells (DK index), and the ratio of the SI obtained with DK20 compared with PPD (PPD index).
Results: The median SI to DK20 in the urethritis was 12.7 which was significantly higher than the controls (7.6, $p < 0.003$). The median SI to the recall antigen PPD was similar in the urethritis patients (17.4) and the controls (22.4). All urethritis patient subgroups had a significantly higher DK index and PPD index than the controls. There was no difference in the PPD and DK index between urethritis naive and urethritis experienced patients and between the culture positive and culture negative urethritis subgroups. In PID patients only the PPD index was significantly higher than the controls.
Conclusion: Men presenting with urethritis and women presenting with PID both have significantly greater peripheral blood mononuclear cell proliferative responses to the DK20 strain of *C trachomatis* than controls. A similar T cell proliferative response pattern in urethritis naive patients with either gonococcal or non-gonococcal urethritis could be because low sensitivity of CT culture failed to diagnose some cases of *C trachomatis*. However, it may also signify earlier exposure of the patients to chlamydial antigens (for example, *C pneumoniae*), cross reacting antigens such as heat shock proteins from other microbial species, or a "bystander" activation of chlamydia specific memory T cells trafficking through mucosal lymphoid tissue during urethritis. These results suggest evidence of T cell mediated response to *C trachomatis* cannot be used as a diagnostic tool.
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Keywords: non-gonococcal urethritis; *Chlamydia trachomatis*; lymphocyte proliferation

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Introduction

Chlamydia trachomatis is an obligate intracellular organism and accounts for 30–60% of cases of non-gonococcal urethritis in men.[1] Antibodies to surface exposed antigens provide some degree of protective immunity, but this appears to be short lived in view of the frequency of reinfection. To clear chlamydial infection, cell mediated immune responses are critical, with an important role for CD4+ lymphocytes, particularly those of the T helper 1 (Th-1) subset which produce interferon γ (IFN-γ).[2–4] A possible role for CD8+ T cells has been proposed, again associated with IFN-γ production.[5]

However, release of IFN-γ, and the associated proinflammatory cytokines and chemokines also produced by chlamydia specific CD4+ and CD8+ T lymphocytes, has a cost.[6 7] By recruiting and activating macrophages, these immune responses can contribute to tissue damage and fibrosis. In the Fallopian tube the inflammatory process results in severe scarring and infertility; this can occur even in asymptomatic patients, and after apparently successful treatment of the infection.[8] Similarly the conjunctival scarring in endemic chlamydial conjunctivitis is thought to be immunologically mediated.[9 10]

While the majority of patients with non-gonococcal urethritis (NGU) respond clinically to tetracycline or erythromycin,[11] and local scarring does not seem to occur, some patients are unresponsive to treatment (persistent NGU) or the problem recurs after a disease free interval (recurrent NGU).[12] We have previously shown that the chemotactic activity of the urethral exudate temporarily declines after treatment in *C trachomatis* positive urethritis but returns to pretreatment levels at 4–6 weeks after the end of therapy in the absence of evidence of reinfection.[13] Chemo-

Table 1 Age and previous history of urethritis in patients presenting with urethritis (n=102) or pelvic inflammatory disease

Condition	*Median age*	*Number*	*Naive†*	*Experienced‡*
N gonorrhoeae urethritis	27	13	8	5
C trachomatis urethritis	30	21	10	11
Urethritis with both organisms	25*	15	10	5
Urethritis with no organism	31	53	19	34
PID	25**	23	18	5
Control	29	37	—	—

*p<0.05 compared with controls, Mann–Whitney.
**p<0.003 compared with controls, Mann–Whitney.
†Naive denotes no previous history of *N gonorrhoeae* or *C trachomatis* infection.
††Experienced patients have documented infection with *N gonorrhoeae*, *C trachomatis*, or NGU on one or more occasions.

tactic activity of the urethra does not significantly decline after treatment of *C trachomatis* negative urethritis. A persisting immunological phenomenon triggered initially by a chlamydial infection has been proposed to explain the persisting inflammation in some patients who do not respond to appropriate antimicrobial therapy as well as the inflammatory process in some patients with *C trachomatis* negative urethritis.[14 15]

Little information is available on T cell proliferative responses to chlamydial antigens in patients with NGU. The aim of the present study was to measure peripheral blood mononuclear cell (PBMC) proliferative responses to *C trachomatis* elementary bodies in males with gonococcal and non-gonococcal urethritis, and in women with a clinical diagnosis of pelvic inflammatory disease (PID), and to compare them with controls. We were also interested to compare the PBMC proliferative response in patients who presented with urethritis for the first time with those who had documented urethritis in the past.

Patients and methods

Patients attending the genitourinary (GUM) clinic with acute urethritis were recruited after informed consent. Urethritis was diagnosed as described.[16] *C trachomatis* and *Neisseria gonorrhoeae* were isolated by culture of urethral swabs.[13] Culture would underestimate the isolation of *C trachomatis.* Clinical and microbiological details of the 102 men presenting with acute urethritis are given in table 1. They were divided according to organisms isolated from the urethra (*C trachomatis* or *N gonorrhoeae*), and whether it was their first (urethritis naive) or subsequent (urethritis experienced) attack. Twenty three women presenting to the GUM clinic with a clinical diagnosis of PID were also investigated. Diagnosis of PID was made on clinical criteria of lower abdominal pain, dyspareunia, adnexal tenderness, cervical excitation, and improvement after 2 weeks of appropriate antimicrobial therapy. Confirmatory laparoscopy was not performed. *C trachomatis* was isolated from the cervical swab in two patients, gonorrhoea from one, and both organisms from another.

Controls were 21 men and 16 non-pregnant women from among laboratory and clinical staff without present or past history of clinical urethritis or chlamydial infection. The controls did not undergo urethral tests or infection testing. Ethics committee approval was obtained for the study.

PROLIFERATION ASSAYS

We measured proliferative responses to *C trachomatis* strain DK20 (an oculogenital strain, serovar E), a lysate of McCoy cells (used to propagate chlamydiae), and the recall antigen purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statenserumsinstitut, Copenhagen, Denmark). *C trachomatis* DK20 was grown in McCoy cells, sonicated, purified by centrifugation over Urografin (Schering Health Care Ltd, UK) and used at a concentration of 1×10^6 infection forming units/ml. Uninfected McCoy cells were grown, sonicated and purified in parallel with the DK20, and used at an equivalent dilution to the *C trachomatis* preparation. Material from McCoy cells was not purified on Urografin since this resulted in the loss of all of the material; thus the concentration of cellular antigens in this control preparation greatly exceeds the concentration in the *C trachomatis* Epstein–Barr preparation.

PBMC were isolated from 20 ml of heparinised blood samples, taken at the same time as samples for syphilis serology. Samples were diluted 1:1 with phosphate buffered saline (PBS), layered onto Ficoll-Paque (Pharmacia Biotech AB, Milton Keynes), and centrifuged for 30 minutes. Cells obtained from the Ficoll/medium interface were harvested and then washed three times. Aliquots of 10^5 cells were then co-cultured in triplicate with the appropriate antigens in 96 U bottomed plates in a final volume of 200 µl in RPMI-1640 medium (Gibco BRL, Paisley) supplemented with 5% heat inactivated AB+ serum, 2 mM L glutamine + 100 µl/ml penicillin + 100 µl/ml streptomycin (Sigma), 1% non-essential amino acids (Sigma), 1% HEPES buffer (Sigma), and 1% sodium pyruvate (Sigma).

The cells were cultured for 6 days at 37°C in 5% carbon dioxide, adding 0.15 µCi tritiated thymidine (Amersham, Aylesbury) to each well for the final 16 hours of culture. The cells were then harvested and thymidine incorporation measured using a liquid scintillation counter. Results were recorded as mean counts per minute (cpm) (SE) and are presented as stimulation indices (SI), where SI = (cpm of cells + antigen)/(cpm of cells only). Results were also expressed in two other ways. Firstly, the ratio of the SI obtained with DK20 compared with McCoy cells was calculated (DK index), as an indication of the specific response to chlamydial antigens as distinct from any antigens originating from the McCoy cells. Secondly, the ratio of the SI obtained with DK20 compared with PPD ratio (PPD index) was calculated, using PPD as a "standard" recall antigen. This index corrects individual subjects' differing ability to mount PBMC proliferative responses to recall antigens in vitro.

STATISTICAL ANALYSIS

The Mann–Whitney test was used to compare proliferative responses between groups.

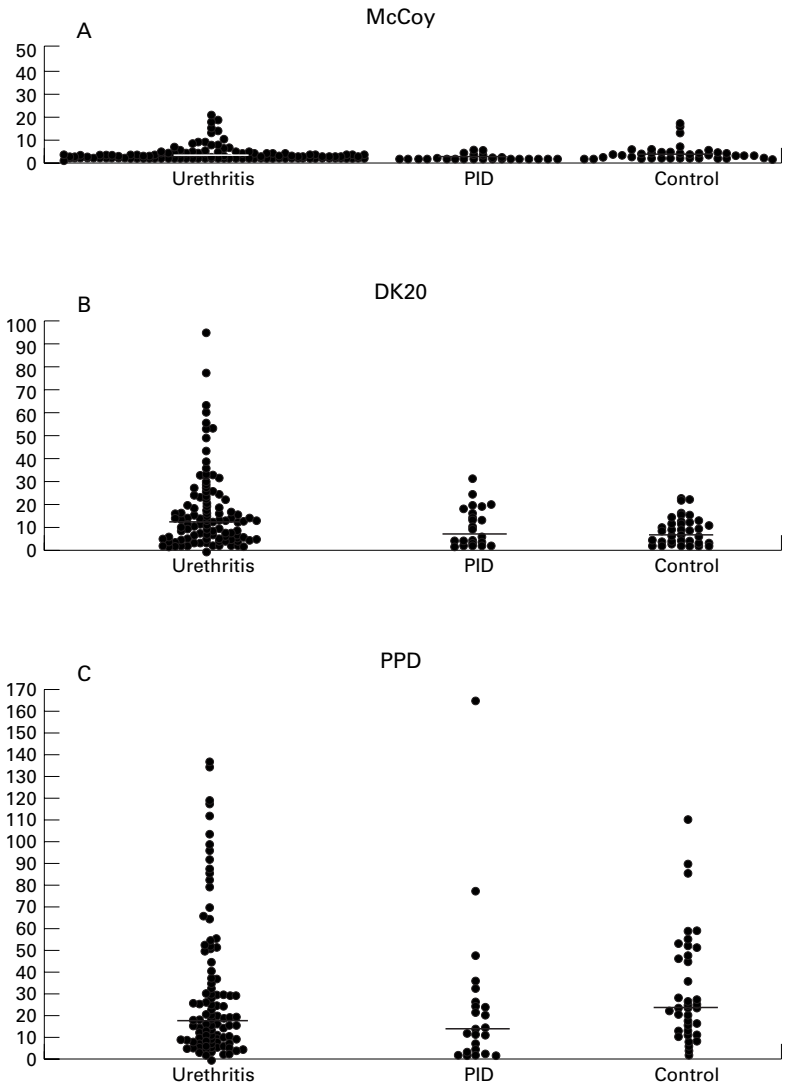

Figure 1 Individual stimulation indices (SI) (A) of peripheral blood mononuclear cell proliferative response to lysate of McCoy cells, DK20 strain of C trachomatis (B), and purified protein derivative (PPD) of Mycobacterium tuberculosis (C) in 102 patients with urethritis, 23 women with a clinical diagnosis of pelvic inflammatory disease (PID), and 37 controls. The lines shows median SI. Note different scales on y axis.

Table 2 Peripheral blood mononuclear cell proliferative response to DK20 strain of C trachomatis in patients with urethritis and pelvic inflammatory disease (PID). Results are expressed as the median ratio of the response to DK 20 and McCoy cells (DK index) and also as the median ratio of response to DK20 and purified protein derivative (PPD) of Mycobacterium tuberculosis (PPD index). A McCoy control was not used in every experiment

	DK index (number)	*Mann–Whitney**	*PPD index (number)*	*Mann–Whitney**
Control	2.3 (37)		0.26 (37)	
All urethritis	6.5 (88)	0.0001	0.57 (102)	0.00001
Urethritis naive†	6.4 (38)	0.014	0.57 (47)	0.003
Urethritis experienced	7.1 (50)	0.04	0.71 (55)	0.001
Chlamydia positive urethritis‡	6.5 (32)	0.02	0.69 (36)	0.00001
Chlamydia negative urethritis	6.5 (56)	0.035	0.51 (66)	0.0001
Chlamydia experienced¶	6.0 (55)	0.006	0.69 (61)	0.00001
Chlamydia naive	6.5 (33)	0.01	0.54 (41)	0.001
Chlamydia positive urethritis naive§	7.0 (17)	0.04	0.74 (20)	0.0002
Chlamydia positive urethritis experienced§	6.0 (16)	NS	0.67 (16)	0.0001
Chlamydia negative urethritis naive§	6.4 (21)	0.05	0.57 (27)	0.005
Chlamydia negative urethritis experienced§	7.1 (34)	0.005	0.5 (39)	0.0001
Gonorrhoea naive	8.4 (6)	0.037	0.74 (8)	0.0007
PID	4.4 (23)	NS	0.51 (23)	0.004

*p value compared with controls.
†Patients presenting for the first time with urethritis.
‡*C trachomatis* during current attack.
¶Urethritis with *C trachomatis* infection at some time.
§Subdivided as to whether *C trachomatis* was isolated or not and whether this was their first episode of clinical urethritis (urethritis naive) or they had a documented history of urethritis (urethritis experienced). Includes coinfection with *N gonorrhoeae*.

Results

Data on age, previous history of urethritis, and genital culture results in the patient groups and controls are given in table 1. Individual SI from the urethritis, PID, and controls to DK20, PPD, and McCoy cells are shown in figure 1. It will be seen that median response to DK20 was higher in the urethritis group (12.7) but not the PID (8.5) compared with the controls (7.6, p = 0.003). However a number of individuals in the control group showed proliferative responses to chlamydial antigens similar to the patients. Median stimulation index to PPD in the control patients (22.4) was significantly higher than that in the patients with PID (12.9 p <0.02) but not urethritis (17.4). The majority of subjects showed a low SI when tested with extracts of McCoy cells compared with their responses to chlamydiae or other recall antigens.

All patient groups had a significantly greater PBMC proliferative response to chlamydial antigens than the controls, as judged by the PPD index. All but the PID group also had a significantly higher DK index compared with the controls (table 2). The median DK index in the *C trachomatis* positive urethritis experienced men, though similar to other urethritis groups (6.0), did not reach statistical significance compared with the controls, perhaps because of the small number of samples tested. There were no differences between the various subgroups, including the comparison between patients with urethritis and documented exposure to *C trachomatis* infection in the past and those with no history of exposure to *C trachomatis*. The proliferative response in the urethritis naive patients was similar irrespective of the urethral microbiology, but was significantly greater than in control subjects regardless of whether *C trachomatis* or *N gonorrhoeae* was isolated or whether no organism was identified. *C trachomatis* positive and *C trachomatis* negative patients had DK and PPD indices not statistically different from those patients with PID.

Discussion

We have shown that men presenting with urethritis and women presenting with a clinical diagnosis of PID have a significantly greater PBMC proliferative response to the DK20 strain of *C trachomatis* than controls who had never experienced urethritis. However, urethritis patients who were known to have previously been exposed to *C trachomatis* did not have a significantly different proliferative response from urethritis patients who had never had a documented exposure to *C trachomatis*. Moreover, patients presenting with urethritis for the first time (urethritis naive) also had similar proliferative responses regardless of whether *C trachomatis*, *N gonorrhoeae*, or no organisms were isolated; in each of these groups the response to *C trachomatis* antigens was significantly greater than in control subjects. Controls were recruited from both sexes, and this could potentially introduce a bias through sexual difference in T cell proliferative re-

sponses. However, in our study the response in women with PID and men with urethritis was similar and significantly greater than the controls.

These results suggest that evidence of T cell mediated responses to *C trachomatis* cannot be used diagnostically to distinguish patients with *C trachomatis* induced urethritis or PID from those in which disease is induced by other organisms. There are few previous studies of PBMC proliferative responses to *C trachomatis* elementary bodies in NGU. Hanna *et al*,[17] in a unselected group of volunteers, found a strong correlation between the presence of antibodies to *C trachomatis* and the lymphocyte proliferative response, though the presence of antibody did not predict lymphocyte reactivity in a given individual. Measurement of antichlamydial antibodies might have identified those patients in our study previously exposed to chlamydial antigens but it may be difficult to distinguish those exposed to *C trachomatis* and *C pneumoniae*. Brunham *et al* [18] found significantly greater lymphocyte transformation to chlamydial elementary bodies in *C trachomatis* positive compared with *C trachomatis* negative men with NGU. They also found that SI to *C trachomatis* was below 3.5 in sexually inexperienced adults and 11 of 12 people attending an STD clinic with neither culture or antibody evidence of exposure to *C trachomatis*. The T cell proliferative response is both serovar specific and against common conserved major outer membrane proteins (MOMP) antigens.[19] Our results suggest that T cell response to chlamydial antigens may be common within this patient population, even when there was no previous clinical history of possible exposure ("urethritis naive") or previously documented *C trachomatis* infection ("chlamydia naive"). Likewise, although GUM patients as a population could be distinguished from controls by their higher responses to *C trachomatis*, such responses were by no means uncommon in the control group. A number of explanations can be put forward to account for these findings.

Culture is known to underestimate *C trachomatis* infection by 30–50%,[20] and would lead to an overestimation of the *C trachomatis* negative groups in our study. However, we found a significantly higher proliferative index in eight patients presenting with gonorrhoea without *C trachomatis* for the first time. In our clinic coinfection with *C trachomatis* of patients with gonorrhoea is 25% by culture and 31.6% by ligase chain reaction (LCR) (Shahmanesh M, unpublished observation). Therefore, an undetected coinfection with *C trachomatis* is unlikely to account for the large difference from the controls in this relatively small number of patients. However, since patients attending STD clinics are more likely to have exposure to sexually transmitted organisms, many of the *C trachomatis* negative patients may have previously experienced subclinical infection by *C trachomatis* which they had subsequently cleared.

Previous infection by *C pneumoniae*, which has several antigens with marked conservation of sequence, may allow cross reactive antibodies and T cells to be generated.[21 22] Interestingly, Horner *et al* found that a high proportion of NGU patients without antibody to *C trachomatis*, or historical evidence of chlamydial infection, had detectable antibodies to *C trachomatis* hsp60[23] and suggested that antibodies to *C trachomatis* hsp60 might also cross react with hsp60 from other urethral sexually transmitted pathogens such as *Mycoplasma genitalium* and *Ureaplasma urealyticum*. In agreement with this idea, in a study of women attending an STD clinic, antibodies against chlamydial hsp60 correlated with PID, age >20, non-white race, >10 lifetime partners, and current oral contraceptive use, but not with positive *C trachomatis* culture or history of chlamydial infection.[24] Exposure to other common organisms may also cause urethritis. *C trachomatis* negative NGU is often diagnosed in male contacts of women with bacterial vaginosis,[25 26] other common genitourinary infections,[27] and after oro-genital contact.[28] The oral cavity contains over 400 micro-organisms.

The T cell epitope which we defined in *C trachomatis* hsp60 also showed substantial sequence conservation in other organisms, including *Escherichia coli*.[21] Infection with other organisms, or even exposure to normal gut flora, might generate some cross reactive immune responses and account for our findings in *C trachomatis* negative and control subjects. In reactive arthritis due to infection with enteric organisms such as *Yersinia*, *Shigella*, *Campylobacter*, and *Salmonella*, a "reactive" urethritis is well described, and the arthritis is associated with immune responses to hsp60.[29 30]

However, since exposure to *C pneumoniae*, or other ubiquitous bacteria, should have been equally common in controls and urethritis patients, it may be that challenge with agents associated with urethritis elicits a boosted response to conserved antigens such as hsp60. It is also possible that urethritis itself might result in the non-specific "bystander" upregulation of responses by memory T cells which traffic through the mucosal lymphoid tissues, which may include chlamydia specific T cells.

Contributors: Original concept by HG and MS; assays by MB supervised by JSHG and JHP; patients recruited by AS supervised by MS; manuscript written by MS with the other authors.

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